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SUFFIELD REPORT

NO. 376

# THE USE OF 4′-AMINOMETHYL-4, 5′, 8-TRIMETHYLPSORALEN IN THE INACTIVATION OF INFLUENZA A/PR8 (U)

by

S.H. Toenjes[1], R.E. Fulton, L.M. Mofford[2], J. Fildes and V.L. DiNinno

Project No. 16B10

January 1984

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A REPORT OF A SUMMER RESEARCH ASSISTANT PROJECT, 1982 (S.H.T.)

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## ABSTRACT

A procedure which utilizes the property of 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) to bind to single stranded RNA in the presence of long wave ultraviolet light (LWUV) has been developed for the inactivation of influenza A/PR8. At a concentration of AMT of 10 µg/ml, inactivation was complete after an exposure time to LWUV of three min. Virus infectivity was not significantly affected by treatment with either AMT or LWUV alone. The ability of influenza A/PR8 to haemagglutinate rooster erythrocytes was unaffected by AMT-LWUV treatment. This procedure provides an alternative to chemical inactivation procedures which can alter the antigenicity of virus preparations and thereby compromise their value as protective immunogens.

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## INTRODUCTION

Inactivation of infectious virus in a killed vaccine must be effected in such a way that antigenicity of the virus remains intact. Any slight changes in antigenic structure induced by the inactivation process can change the specificity of the antibodies produced by the host and thereby reduce or eliminate their effectiveness (Webster and Laver, 1972). An inactivation procedure which depends upon inactivation of nucleic acid would therefore be superior to one that acts by denaturation of coat proteins. Such a procedure, which utilizes the property of psoralen derivatives to bind to single stranded RNA in the presence of long wave ultraviolet light, has been described for the inactivation of RNA viruses (Nakashima et al., 1979; Hansen

et al., 1977; Redfield et al., 1981; Hearst and Thirty, 1977). The binding of psoralen and its derivatives to virus RNA serves to prevent the replication of the viral genome once the virus has infected the cell. Psoralen binding and crosslinking mechanics and kinetics have been reviewed by Isaacs et al., (1977).

This present study was undertaken to develop a method, using psoralen, for the inactivation of Influenza A/PR8 virus. Once established, this method could be used in further studies projected to compare protective levels of immunity in mice immunized with live attenuated and inactivated virus, respectively, both by conventional routes of immunization (intravenous, intraperitoneal, intramuscular) and by aerosolization. This study constitutes a continuation of work initiated during the summer of 1981.

## MATERIALS AND METHODS

A psoralen derivative, 4'-aminomethyl-4, 5', 8-trimethylpsoralen hydrochloride (AMT) (Calbiochem-Behring, LaJolla, California), was used in these trials. The synthesis and analytical specifications of this compound are described by Isaacs et al., 1977. AMT was dissolved in dimethylsulfoxide (DMSO) to a concentration of 1000 ug/ml. Stock solutions of 1, 10 and 100 ug/ml were made from this using phosphate-buffered saline (PBS) (Oxoid, Med-Ox Chemicals, Ottawa, Ontario) as the diluent. According to the instructions of the manufacturer, all solutions were stored in brown glass bottles and kept at -70°C to protect them from light. A modification of the method of Redfield et al., 1981, was used to inactivate the virus, as follows.

A 100 ul volume of AMT stock solution was mixed with 900 µl of virus suspension (crude preparation of Influenza A/PR8 grown in egg allantoic fluid). The virus sample had previously been tested for haemagglutination

(HA) and infectivity and was found to contain approximately 256 HA units per 25 µl and 5 x $10^6$ plaque-forming units (pfu)/ml, respectively. This sample was subsequently aliquoted into 900 µl volumes and stored at -70°C until such time as it was needed.

AMT-virus samples were then placed in 60 x 15 mm Contur$^R$ culture dishes (Lux Scientific Corporation, Newbury Park, California) and the covers replaced. The dishes were tilted to ensure even spreading of the samples on the plastic and then placed inside a box containing a long-wave ultraviolet (LWUV) light source (Ultraviolet Products Incoporated, San Gabriel, California). The lamp was operated for the prescribed period of LWUV exposure after which samples were removed and pipetted into sterile polystyrene tubes (Fisherbrand Catalogue No. 14-956-3D, Fisher Scientific Company, Pittsburgh, Pennsylvania).

The samples were then assayed for HA by haemagglutination assay and for infectivity by influenza plaque assay. In some cases this was done with samples that had been frozen and then thawed while in others these tests were done immediately after inactivation with AMT and exposure to LWUV. Microtitre plates (U-shaped, 8 x 12 wells per flat) were used for performance of HA assays. Dilutions were made in PBS, using a Titertek$^R$ (TM) microdiluter calibrated to deliver 25 ul (Flow Laboratories, Mississauga, Ontario). A suspension of 0.75% rooster red blood cells (Institut Armand Frappier, Laval, Quebec) was prepared in PBS. The assay procedure was that described by Fulton, 1981.

Plaque assays were performed using a continuous line of African Green monkey kidney cells (Vero). Cell monolayers were grown to confluency in 60 x 15 mm Contur$^R$ culture dishes and infected 22 to 26 hours after seeding. Ten-fold dilutions of virus-AMT samples were prepared in PBS and volumes of 100 ul were added to each cell monolayer after they had been washed once with

PBS. Adsorption for one hour at 37°C was followed by addition of 5 ml of overlay medium per culture dish, as described by Tobita et al., 1975 and as given in the Appendix. The medium was allowed to solidify at room temperature after which the plates were incubated at 37°C for a period of five to six days (depending on whether the fifth day fell on a weekday or a Sunday). Each plate was then overlayed with three ml of fixing solution (ethanol: acetic acid: triple distilled water = 9:2:9). After fifteen minutes the agar overlays were removed and the fixed cell monolayers washed with tap water. To stain the monolayers, crystal violet (1% in distilled water) was applied to each dish, then washed off with tap water. The dishes were air dried and plaques counted using a lightbox and the naked eye.

## RESULTS

In order to determine the relationship of AMT concentration to the LWUV exposure time required to effect inactivation of the virus, the following pilot experiment was performed.

| Concentration of AMT (μg/ml) | Time of Exposure to LWUV (min) |
|---|---|
| 0.1 | 15, 30, 60 |
| 1.0 | 15, 30, 60 |
| 10.0 | 15, 30, 60 |

The following plaque assay results were obtained.

| Concentration of AMT (µg/ml) | Time of Exposure to LWUV (min) | Infectivity (pfu/ml) ± Standard Error (SE) |
|---|---|---|
| 0.0 | 0 | $5.15 \times 10^5$ (±.35) |
| 0.0 | 60 | $5.40 \times 10^5$ (±.70) |
| 0.1 | 15 | $8.50 \times 10^4$ (±.10) |
| 0.1 | 30 | N/A |
| 0.1 | 60 | N/A |
| 1.0 | 15, 30, 60 | - |
| 10.0 | 15, 30, 60 | - |

N/A = titre not available

- = no plaques at $10^0$ dilution

At 0.1 µg/ml AMT and exposure times of 30 and 60 min, respectively, samples were not diluted sufficiently to obtain end-point infectivity titres. However, the number of plaques obtained at $10^{-1}$ dilution at the 30 and 60 min exposure times was the same as, or similar to, the number obtained at the 15 min exposure. Therefore, we concluded that the infectivity remaining after 30 and 60 min of exposure to LWUV was still quite high and was in the order of magnitude of that observed after 15 min of exposure.

The HA titre obtained on samples treated with 0.1, 1.0 and 10 µg/ml AMT, respectively, was the same (1024 HA units/25 µl) regardless of whether the exposure time to LWUV had been 15, 30 or 60 min. In addition, control samples which had not been treated with AMT but had been exposed to LWUV for 60 min also had a titre of 1024 units/25 µl.

In order to verify these findings, the above experiment was repeated. The findings were similar. No plaques were obtained in virus suspensions treated with either 1.0 or 10 µg/ml AMT and exposed to LWUV for 15 min or

longer, while the titre obtained at 0.1 µg/ml was the same regardless of whether the LWUV exposure had been 15, 30 or 60 min. It was therefore concluded that a concentration of 0.1 µg/ml AMT would be unsuitable, and that in further experiments 1.0 and 10 µg/ml AMT should be evaluated in combination with shorter exposure times to LWUV. Accordingly, the following trial schedule of inactivation was adopted.

| Concentration of AMT (µg/ml) | Time of Exposure to LWUV (min) |
|---|---|
| 1.0 | 0.167, 1.0, 5.0, 15.0 |
| 10.0 | 0.167, 1.0, 5.0, 15.0 |

The following data was obtained.

| Concentration of AMT (µg/ml) | Time of Exposure to LWUV (min) | Infectivity (pfu/ml) ± S.E. | Surviving Fraction Infectivity | HA |
|---|---|---|---|---|
| 0.0 | 0.000 | $1.06 \times 10^6$ (±.02) | 1.0000 | $2^8$ |
| 1.0 | 0.167 | $9.90 \times 10^5$ (±3.4) | 0.9384 | $2^7$ |
| 1.0 | 1.000 | $3.15 \times 10^5$ (±0.65) | 0.2986 | $2^7$ |
| 1.0 | 5.000 and 15.000 | - | | $2^7$ |
| 10.0 | 0.167 | $2.25 \times 10^5$ (±0.85) | 0.2133 | $2^7$ |
| 10.0 | 1.000, 5.000 and 15.000 | - | | $2^8$ |

- = no plaques seen at $10^0$ dilution.

This experiment was repeated and the following results obtained.

| Concentration of AMT (µg/ml) | Time of Exposure to LWUV (min) | Infectivity (pfu/ml) ± S.E. | Surviving Fraction Infectivity | HA |
|---|---|---|---|---|
| 0.0 | 0.000 | $7.50 \times 10^6$ (±.40) | 1.0000 | N/A |
| 1.0 | 0.167 | $2.20 \times 10^6$ (±.10) | 0.2330 | N/A |
| 1.0 | 1.000 | $7.80 \times 10^5$ (±.10) | 0.1040 | |
| 1.0 | 5.000 | $1.10 \times 10^4$ (±.02) | 0.0013 | |
| 1.0 | 15.000 | $4.30 \times 10^3$ (±.05) | 0.0006 | |
| 10.0 | 0.167 | $1.61 \times 10^5$ (±.20) | 0.0214 | |
| 10.0 | 1.000 | $8.15 \times 10^3$ (±.35) | 0.0011 | |
| 10.0 | 5.000 and 15.000 | - | - | |

N/A = titre not available

- = no plaques at $10^0$ dilution

Data obtained on the repeat experiment indicated higher survival rates at 1.0 µg/ml AMT and exposure times of 5 and 15 min, respectively, as well as at 10 µg/ml AMT and an exposure time of 1.0 min. This discrepancy, however, was explained by the fact that the titre of the original (untreated) virus sample was much higher. This finding emphasized the need for standardization between plaque assays as well as within them. Therefore, a large volume of stock virus suspension was aliquoted into 900 µl volumes, stored at -70°C and used as a standard for the duration of the trials.

The findings indicated that a much more rigorous schedule of long wave ultraviolet light exposures would be necessary to produce an inactivation curve. As well, in order to determine whether freezing and thawing of the virus samples affected the results, treated and LWUV exposed samples were assayed both immediately and after one cycle of freezing and thawing. The following are the results of two inactivation trials in which these parameters were evaluated.

TRIAL 1

ASSAYED IMMEDIATELY

| Concentration of AMT (μg/ml) | Time of Exposure to LWUV (min) | Infectivity (pfu/ml) ± S.E. | Surviving Fraction Infectivity | HA |
|---|---|---|---|---|
| 0.0 | 0.000 | $6.65 \times 10^5$ (±.65) | 1.000 | 256 |
| 0.0 | 5.000 | $5.70 \times 10^5$ (±.30) | 0.857 | 256 |
| 1.0 | 0.000 | $5.35 \times 10^5$ (±.05) | 0.805 | 128 |
| 1.0 | 0.167 | $5.53 \times 10^5$ (±.15) | 0.832 | 256 |
| 1.0 | 1.000 | $2.35 \times 10^5$ (±.38) | 0.353 | 512 |
| 1.0 | 2.000 | $3.90 \times 10^4$ (±.05) | 0.059 | 512 |
| 1.0 | 3.000 | $2.97 \times 10^4$ (±.00) | 0.045 | 128 |
| 1.0 | 4.000 | $1.30 \times 10^4$ (±.80) | 0.020 | 2048 |
| 1.0 | 5.000 | - | - | - |
| 10.0 | 0.000 | $6.65 \times 10^5$ (±1.85) | 1.030 | 32 |
| 10.0 | 0.167 | $2.07 \times 10^5$ (±.06) | 0.311 | 256 |
| 10.0 | 0.333 | $3.08 \times 10^4$ (±.79) | 0.046 | 512 |
| 10.0 | 0.500 | $1.72 \times 10^4$ (±.27) | 0.026 | 512 |
| 10.0 | 0.667 | $6.40 \times 10^3$ (±1.30) | 0.004 | 128 |
| 10.0 | 1.000 | - | - | - |
| ASSAYED AFTER ONE CYCLE OF FREEZING AND THAWING | | | | |
| 0.0 | 0.000 | $2.70 \times 10^6$ (±.22) | 1.000 | 256 |
| 0.0 | 5.000 | $2.33 \times 10^6$ (±.06) | 0.863 | 128 |
| 1.0 | 0.000 | $1.47 \times 10^6$ (±.01) | 0.543 | 64 |
| 1.0 | 0.167 | $1.84 \times 10^6$ (±.01) | 0.680 | 128 |
| 1.0 | 1.000 | $8.10 \times 10^5$ (±.60) | 0.300 | 128 |
| 1.0 | 2.000 | $1.20 \times 10^5$ (±.03) | 0.044 | 64 |
| 1.0 | 3.000 | $8.40 \times 10^4$ (±.50) | 0.031 | 128 |
| 1.0 | 4.000 | $2.28 \times 10^4$ (±.30) | 0.008 | 128 |
| 1.0 | 5.000 | $1.97 \times 10^4$ (±.07) | 0.007 | 128 |
| 10.0 | 0.000 | $1.00 \times 10^6$ (±.02) | 0.370 | 128 |
| 10.0 | 0.167 | $2.68 \times 10^5$ (±.19) | 0.099 | 256 |
| 10.0 | 0.333 | $6.05 \times 10^4$ (±.65) | 0.022 | 256 |
| 10.0 | 0.500 | $1.95 \times 10^4$ (±.15) | 0.007 | 128 |
| 10.0 | 0.667 | $1.32 \times 10^4$ (±.01) | 0.005 | 128 |
| 10.0 | 1.000 | $1.11 \times 10^4$ (±.21) | 0.004 | 128 |

- = no plaques at $10^0$ dilution

TRIAL 2

ASSAYED IMMEDIATELY

| Concentration of AMT (µg/ml) | Time of Exposure to LWUV (min) | Infectivity (pfu/ml) ± S.E. | Surviving Fraction Infectivity | HA |
|---|---|---|---|---|
| 0.0 | 0.000 | $4.68 \times 10^6$ (±.18) | 1.000 | N/A |
| 0.0 | 5.000 | $4.35 \times 10^6$ (±.25) | 0.930 | |
| 1.0 | 0.000 | $4.85 \times 10^6$ (±.05) | 1.036 | |
| 1.0 | 0.167 | $4.90 \times 10^6$ (±.20) | 1.070 | |
| 1.0 | 1.000 | $7.55 \times 10^5$ (±.85) | 0.161 | |
| 1.0 | 2.000 | $2.45 \times 10^5$ (±.60) | 0.052 | |
| 1.0 | 3.000 | $1.12 \times 10^5$ (±.14) | 0.024 | |
| 1.0 | 4.000 | $7.30 \times 10^4$ (±.50) | 0.016 | |
| 1.0 | 5.000 | $4.75 \times 10^4$ (±.45) | 0.015 | |
| 10.0 | 0.000 | $2.95 \times 10^6$ (±.75) | 0.630 | |
| 10.0 | 0.167 | $1.17 \times 10^6$ (±.11) | 0.249 | |
| 10.0 | 0.333 | N/A | N/A | |
| 10.0 | 0.500 | $1.59 \times 10^5$ (±.06) | 0.034 | |
| 10.0 | 0.667 | $6.70 \times 10^4$ (±.11) | 0.014 | |
| 10.0 | 1.000 | $2.08 \times 10^4$ (±.22) | 0.004 | |
| 10.0 | 2.000 | $3.60 \times 10^2$ (±.30) | 0.000 | |
| 10.0 | 3.000 | $9.50 \times 10^1$ (±.15) | 0.000 | |
| ASSAYED AFTER ONE CYCLE OF FREEZING AND THAWING | | | | |
| 0.0 | 0.000 | $5.27 \times 10^6$ (±.27) | 1.000 | 256 |
| 0.0 | 5.000 | $2.65 \times 10^6$ (±.15) | 0.503 | 128 |
| 1.0 | 0.000 | $7.05 \times 10^6$ (±.05) | 1.339 | 256 |
| 1.0 | 0.167 | $3.50 \times 10^6$ (±.50) | 0.665 | 512 |
| 1.0 | 1.000 | $6.60 \times 10^6$ (±.60) | 0.125 | 256 |
| 1.0 | 2.000 | $1.65 \times 10^5$ (±.15) | 0.031 | 256 |
| 1.0 | 3.000 | $7.05 \times 10^4$ (±.75) | 0.013 | 512 |
| 1.0 | 4.000 | $2.80 \times 10^4$ (±.50) | 0.005 | 512 |
| 1.0 | 5.000 | $2.70 \times 10^4$ (±.10) | 0.005 | 512 |
| 10.0 | 0.000 | $3.00 \times 10^6$ (±.10) | 0.570 | 512 |
| 10.0 | 0.167 | $7.80 \times 10^5$ (±.50) | 0.148 | 512 |
| 10.0 | 0.333 | $3.00 \times 10^6$ (±.60) | 0.570 | 512 |
| 10.0 | 0.500 | $1.02 \times 10^5$ (±.10) | 0.019 | 256 |
| 10.0 | 0.667 | $1.95 \times 10^4$ (±.35) | 0.004 | 256 |
| 10.0 | 1.000 | $1.26 \times 10^4$ (±.06) | 0.002 | 256 |
| 10.0 | 2.000 | $1.35 \times 10^2$ (±.05) | 0.000 | 256 |
| 10.0 | 3.000 | $3.00 \times 10^1$ (±1.00) | 0.000 | 128 |

N/A = titre not available

STATISTICAL ANALYSIS OF DATA (Hewlett Packard HP-85 computer software)

Two Way Analysis of Variance (test for interaction between AMT and LWUV Light):

TWO WAY ANALYSIS OF VARIANCE
R = 2
C = 2
N = 4

| | ROW | COLUMN | OBSERVATIONS Ln (Surviving Fraction) |
|---|---|---|---|
| No AMT, No LWUV | 1 | 1 | 0.00, 0.00, 0.00, 0.00 |
| 1 µg/ml AMT, No LWUV | 1 | 2 | -0.22, -0.61, 0.04, 0.29 |
| No AMT, 5 min LWUV | 2 | 1 | -0.15, -1.47, -0.07, -0.07 |
| 1 µg/ml AMT, 5 min LWUV | 2 | 2 | -9.13, -5.27, -6.62, -4.20 |

ON ROW 2 COL 2 CHANGE
-9.13 TO -4.92

ANALYSIS OF VARIANCE

| SOURCE/DF | | SS | MS | F |
|---|---|---|---|---|
| TOTAL | 15 | 82.3 | | |
| ROWS | 1 | 31.0 | 31.0 | 75.7 |
| COLS | 1 | 24.4 | 24.4 | 59.5 |
| RXC | 1 | 22.0 | 22.0 | 53.6 |
| ERROR | 12 | 4.9 | 0.4 | |

ROW CONTRAST
1.0000, -1.0000

COMPARISON = 2.786
CONTRAST SS = 31.038
FRATIO = 75.718

COLUMN CONTRAST
1.000, -1.0000

COMPARISON = 2.469
CONTRAST SS = 24.374
F RATIO = 59.460

Conclusions: There is a significant interaction between the two variables in question. There also happens to be a significant difference between rows and columns which brings up a question of this being a result of the interaction only or because of the AMT or LWUV alone. Therefore ANOVA was done checking for each variable separately.

EFFECT OF AMT WITHOUT LWUV ON INFLUENZA A/PR8 INFECTIVITY

ONE WAY ANALYSIS OF VARIANCE

Ln (Surviving Fraction Infectivity)

TRT #1
(0 µg/ml AMT; 0 exposure LWUV)
0.00 0.00 0.00
0.00

TRT #2
(1.0 µg/ml AMT; 0 exposure LWUV)
-.22 -.61 .04
.29

TRT #3
(10.0 µg/ml AMT; 0 exposure LWUV)
.03 -.99 -.46
-.56

| TRT # | N | MEAN | VARIANCE |
|---|---|---|---|
| 1 | 4 | 0.0000 | 0.0000 |
| 2 | 4 | -.1252 | .1482 |
| 3 | 4 | -.4969 | .1763 |

ANALYSIS OF VARIANCE

| SOURCE/DF | | SS | MS | F |
|---|---|---|---|---|
| TOTAL | 11 | 1.5 | | |
| TRTS | 2 | 0.5 | 0.3 | 2.5 |
| ERROR | 9 | 1.0 | 0.1 | |

DF NUM = 2
DF DEN = 9
F = 2.4697
PROB F > 2.4697 = .1396

CONCLUSION: At the level of significance of P = 0.05, by the F-test there is no effect of AMT without LWUV light.

EFFECT OF LWUV WITHOUT AMT ON INFLUENZA A/PR8 INFECTIVITY

ONE WAY ANALYSIS OF VARIANCE

Ln (Surviving Fraction Infectivity)

TRT #1
(0 µg/ml AMT; 0 exposure LWUV)
0.00 0.00 0.00
0.00

TRT #2
(0 µg/ml AMT; 5 min exposure LWUV)
-.15 -.15 -.07
-.69

| TRT # | N | MEAN | VARIANCE |
|---|---|---|---|
| 1 | 4 | -0.0000 | 0.0000 |
| 2 | 4 | -.2653 | .0802 |

ANALYSIS OF VARIANCE

| SOURCE/DF | | SS | MS | F |
|---|---|---|---|---|
| TOTAL | 7 | 0.4 | | |
| TRTS | 1 | 0.1 | 0.1 | 3.5 |
| ERROR | 6 | 0.2 | 0.0 | |

DF NUM = 1
DF DEN = 6
F = 3.5108
PROB F > 3.5108 = .1101

CONCLUSION: AT P = 0.05, by the F-test LWUV no effect of light without AMT could be found.

COMULATIVE DATA AT 1.0 μg/ml,
TOTALS FOR SIX RUNS, TIME FROM
T = 0 TO T = 5 MIN

| I | TIME(MIN) X(I) | Ln (Surviving Fraction Infectivity) Y(I) |
|---|---|---|
| 1 | 0.0000 | 0.0000 |
| 2 | 0.1670 | -0.0636 |
| 3 | 1.0000 | -1.2087 |
| 4 | 0.0000 | 0.0000 |
| 5 | 0.1670 | -1.4567 |
| 6 | 1.0000 | -2.2634 |
| 7 | 5.0000 | -6.6226 |
| 8 | 0.0000 | 0.0000 |
| 9 | 0.1670 | -0.1839 |
| 10 | 1.0000 | -1.0413 |
| 11 | 2.0000 | -2.8370 |
| 12 | 3.0000 | -3.1078 |
| 13 | 4.0000 | -3.9322 |
| 14 | 0.0000 | -0.6114 |
| 15 | 0.1670 | -0.3863 |
| 16 | 1.0000 | -1.2040 |
| 17 | 2.0000 | -3.1145 |
| 18 | 3.0000 | -3.4705 |
| 19 | 4.0000 | -4.7795 |
| 20 | 5.0000 | -4.9226 |
| 21 | 0.0000 | 0.0357 |
| 22 | 0.1670 | 0.0677 |
| 23 | 1.0000 | -1.8245 |
| 24 | 2.0000 | -2.9498 |
| 25 | 3.0000 | -3.7372 |
| 26 | 4.0000 | -4.1605 |
| 27 | 5.0000 | -4.2024 |
| 28 | 0.0000 | 0.2919 |
| 29 | 0.1670 | -0.4083 |
| 30 | 1.0000 | -2.0762 |
| 31 | 2.0000 | -3.4629 |
| 32 | 3.0000 | -4.3132 |
| 33 | 4.0000 | -5.2348 |
| 34 | 5.0000 | -5.2730 |

MAXIMUM DEGREE REGRESSION = 1

BASIC STATISTICS

MEANS, VARIANCES, CORRELATION
X MEAN = 1.8530
VAR(X) = 3.1947
Y MEAN = -2.3075
VAR(Y) = 3.8776

| MINIMUM | MAXIMUM |
|---|---|
| .0000 | 5.0000 |
| -6.6226 | .2919 |

RXY = .9539

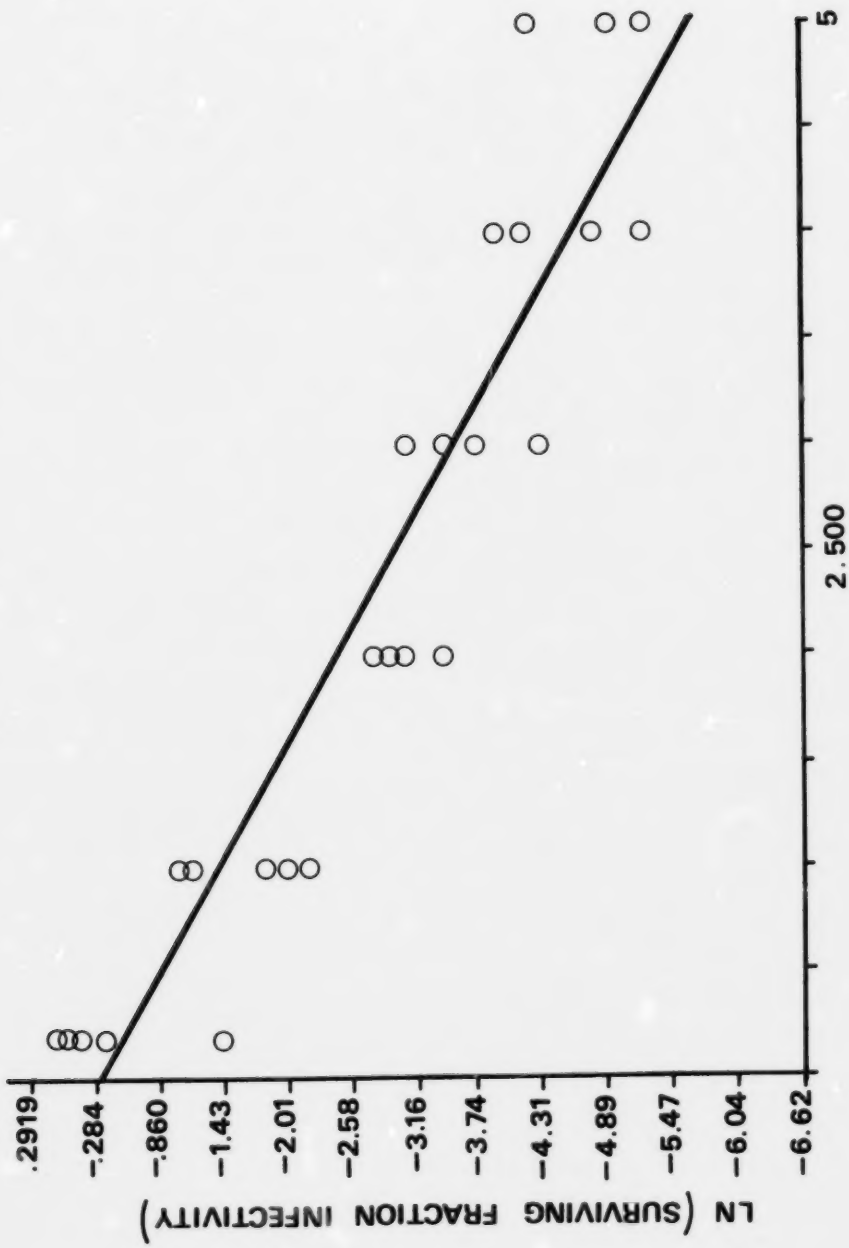
AOV: LINEAR REG: CODE I

| SOURCE/DF | SS | MS | F |
|---|---|---|---|
| TOTAL 33 | 128.0 | | |
| REG 1 | 116.4 | 116.4 | 323.5 |
| RESID 32 | 11.5 | 0.4 | |

R SQUARE = 0.910

YHAT = -0.360+ -1.051 X

CONCLUSION: There is significant regression. The exponential model and the data collected here fit very well. For P = 0.01, and N = 34, the critical value for R is ±0.435. Therefore the value of -0.9539 is very significant.




REGRESSION CURVE RELATING LWUV EXPOSURE TIME TO LN SURVIVING FRACTION INFECTIVITY

AMT at 1.0 μg/mL; exposure time t = 0 to t = 5 min.

COMULATIVE DATA AT 10 μg/ml,
TOTALS FOR SIX RUNS, TIME FROM
T = 0 TO T = 3 MIN

| I | TIME(MIN) X(I) | Ln (Surviving Fraction Infectivity) |
|---|---|---|
| 1 | 0.0000 | 0.0000 |
| 2 | 0.1670 | -1.5451 |
| 3 | 0.0000 | 0.0000 |
| 4 | 0.1670 | -3.8444 |
| 5 | 1.0000 | -6.8243 |
| 6 | 0.0000 | 0.0296 |
| 7 | 0.1670 | -1.1680 |
| 8 | 0.3330 | -0.9009 |
| 9 | 0.5000 | -3.6535 |
| 10 | 0.6670 | -5.6213 |
| 11 | 0.0000 | -0.9932 |
| 12 | 0.1670 | -2.3116 |
| 13 | 0.3330 | -3.7987 |
| 14 | 0.5000 | -4.9337 |
| 15 | 0.6670 | -5.3206 |
| 16 | 1.0000 | -5.4943 |
| 17 | 0.0000 | -0.4616 |
| 18 | 0.1670 | -1.3907 |
| 19 | 0.5000 | -3.3852 |
| 20 | 0.6670 | -4.2461 |
| 21 | 1.0000 | -5.4185 |
| 22 | 2.0000 | -9.4727 |
| 23 | 3.0000 | -10.9049 |
| 24 | 0.0000 | -0.5625 |
| 25 | 0.1670 | -1.9099 |
| 26 | 0.3330 | -2.8651 |
| 27 | 0.5000 | -3.9487 |
| 28 | 0.6670 | -5.5983 |
| 29 | 1.0000 | -6.0390 |
| 30 | 2.0000 | -10.5714 |
| 31 | 3.0000 | -12.0754 |

MAXIMUM DEGREE REGRESSION = 1

BASIC STATISTICS

MEANS, VARIANCES, CORRELATION
X MEAN = 0.0000
VAR(X) = 0.0000
Y MEAN = 0.0000
VAR(Y) = 0.0000

| MINIMUM | MAXIMUM |
|---|---|
| .0000 | 1.0000 |
| -6.8243 | .0296 |

RXY = .94

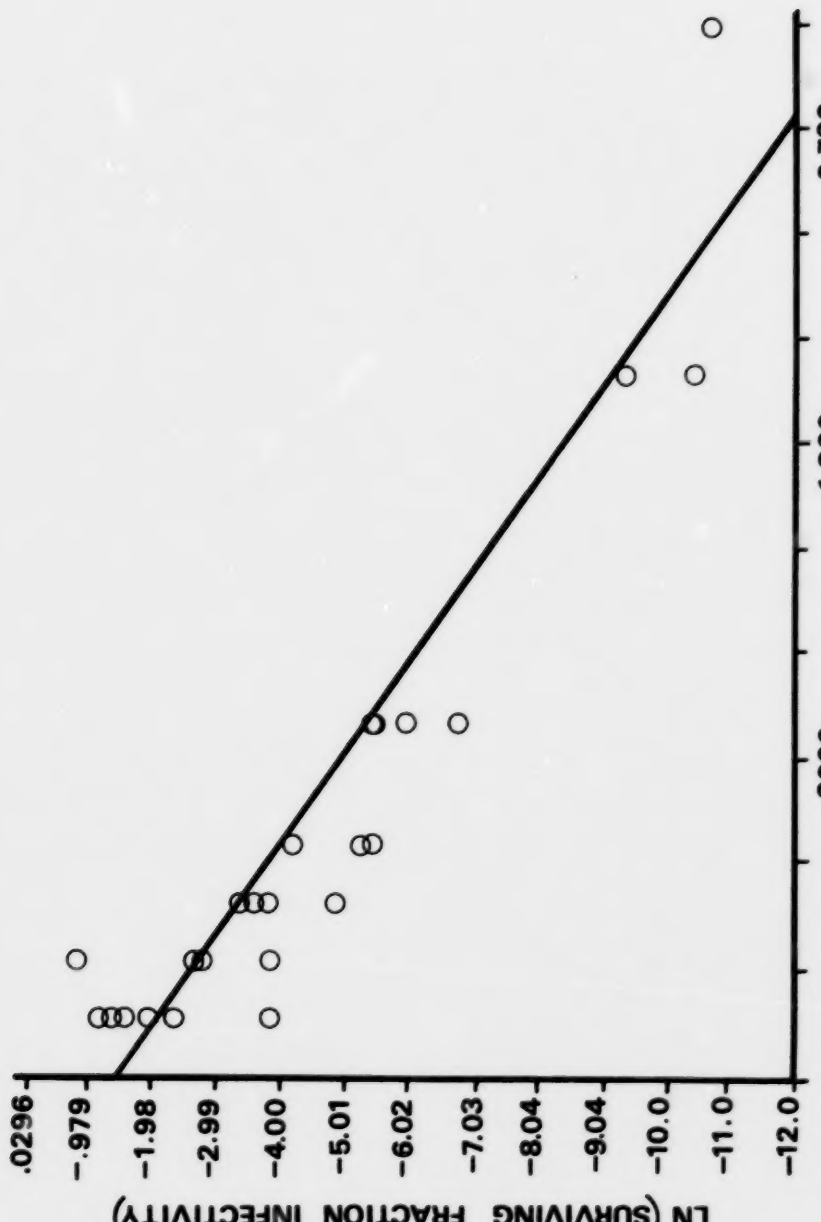
AOV: LINEAR REG: CODE I

| SOURCE/DF | SS | MS | F |
|---|---|---|---|
| TOTAL 30 | 330.0 | | |
| REG 1 | 292.0 | 292.0 | 222.7 |
| RESID 29 | 39.0 | 1.3 | |

R SQUARE = 0.885

YHAT = -1.456+ -3.870 X

CONCLUSION: This represents a significant regression. The critical R value in this case is ±0.456. This means that the R value of 0.94 is very significant.




REGRESSION CURVE RELATING LWUV EXPOSURE TIME TO LN SURVIVING FRACTION INFECTIVITY

AMT at 10 μg/mL; exposure time t = 0 to t = 3 min.

## DISCUSSION

AMT and long-wave ultraviolet light can be used successfully to reduce the infectivity of Influenza A/PR8. Some interesting conclusions can be drawn from the results presented.

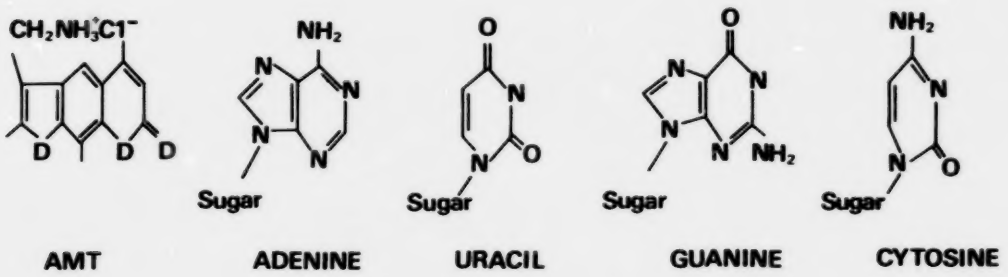
It appears that AMT concentration is too low between 0.1 and 1.0 µg/ml since no great loss in infectivity was noted at 0.1 µg/ml AMT even though the exposure time was up to sixty minutes. From 1.0 to 10 µg/ml AMT however, it is the length of time of LWUV exposure that seems to be the critical variable. The AMT concentration is important here as well but not to such a great extent. This was arrived at since the decay constants at 1.0 and 10 µg/ml for the cumulative data were -1.051 and -3.870, respectively. Suppose the rate of drop in infectivity (ie. the decay constant) was linearly related to AMT concentration as proposed by the exponential curve:

$$Y = N_0 e^{-kt}$$

where $N_0$ is the original infectivity titre, t is the time of LWUV exposure, -k is the decay rate constant and Y is the surviving infectivity after treatment. One would expect the decay rate constant to be proportional to the concentration of AMT. This is inherent in the exponential model which fits to our data very well (correlation coefficients at 1.0 and 10 µg/ml AMT = 0.91 and 0.89, respectively). However this is not the case since the constant at 10 µg/ml AMT is less than four times that at 1.0 µg/ml AMT. This suggests a saturation of binding sites on the RNA which can be explained by the following.

According to Isaacs et al., 1977, there are three reactions which take place during the inactivation. Firstly, an intercalation complex forms between the AMT and nucleic acid. This is not dependent on LWUV but is

dependent on AMT concentration. It involves the juxtapositioning of AMT and single stranded RNA, similar to that found in base pairing of double stranded nucleic acids. This is possibly due to the structural similarity between AMT and nucleotide bases (see diagram below).

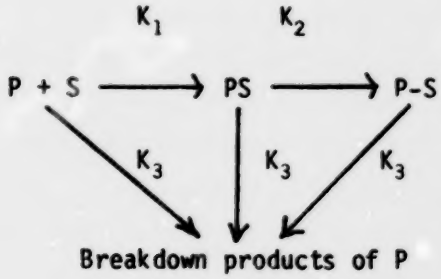



AMT ADENINE URACIL GUANINE CYTOSINE

The second process involved is covalent bonding between RNA and AMT. This involves a wavelength dependent electromagnetic excitation of AMT (absorption maximum approximately 295 nm) (see absorption spectrum of AMT, Appendix). The final reaction is the photodestruction of the psoralen derivative. As previously mentioned, AMT was stored in brown bottles to protect it from light. Since AMT exhibits another absorption peak at 340-350 nm (see Appendix), visible light could activate (excite) the compound and thereby cause it to "photolyse", thereby reducing its effectiveness as a photoreactive compound.

The reactions which take place during inactivation of RNA can be simplified in the following diagram, where P is the psoralen derivative (AMT) and S is the binding site for the AMT. PS represents the intercalation complex where P and S are in close proximity to one another but not yet

bonded, and P-S represents the covalently bonded AMT-RNA complex.




It is my hypothesis that between 0.1 and 1.0 μg/ml AMT, P is rate limiting and therefore rate determining, whereas from 1.0 to 10 μg/ml AMT, $k_1$ reaches a maximum and exposure time to LWUV becomes rate determining.

It should be noted that neither AMT nor LWUV alone had any significant effect on virus infectivity. Samples treated with AMT only, may have exhibited some loss of infectivity due to the presence of long wave ultraviolet light in the fluorescent room lighting. It was not possible, however, to avoid this since the mixing of AMT and virus, as well as the plaque assay, were by necessity performed in the presence of light. More rigorous measures could have been taken to avoid this background inactivation but the small drop in infectivity noticed with some samples was not considered to be significant when compared to the large drop in infectivity after exposure to LWUV.

Antigenicity of the viral preparations was not significantly affected by the AMT-LWUV treatment. Haemagglutination titres were found to be variable, but did not significantly decrease over time. The variable HA endpoints may have been inherent in the test itself. The process of haemagglutination involved the crossbinding of virus and rooster red blood cells, hence any variablility with these reagents might result in variability in

test results. Influenza is known to be variable in its structure as exhibited by a high degree of pleomorphism (Choppin et al., 1961). In addition, rooster blood obtained from Institut Armand Frappier was, at times, poor with respect to cell quality. Sometimes the cells arrived clotted, while at other times the entire bottle contained lysed cells. In addition, variation between birds with respect to the number of virus receptors on the red blood cells should be taken into consideration. Despite the fact that the HA test is widely used as a means of titrating for the presence of hemagglutinating virus, it is sometimes observed to be quite imprecise. For this reason, minor flucuations in HA titre should not be interpreted as being due to the effect of AMT-LWUV on the samples, but should be interpreted as being due to variability within the assay.

It was interesting to note that, occasionally, what appeared to be a prozone effect occured in the plaque assay. In undiluted samples, no plaques were seen, whereas plaques were present at $10^{-1}$ and $10^{-2}$ dilutions. This phenomenon has also been described by Hsuing et al., 1961, who noted that a myxovirus isolated from blood produced approximately fifty plaques at a $10^{-5}$ dilution but failed to produce plaques at stock and $10^{-1}$ dilutions. An explanation for this observation occurring with inactivated virus samples is proposed as follows. Regardless of the infectious titre of a sample, there will be a constant number of virus particles in a specific dilution and sample size of a given preparation. Suppose a sample is tested by plaque assay and yields ten plaques at a $10^{-4}$ dilution. This would be equivalent to a titre of $1.0 \times 10^6$ pfu/ml (volume of inoculum 100 µl). That is, a volume of 100 µl of undiluted sample would contain 100,000 infections virus particles. Now, suppose that this 100 µl sample were inactivated so that only one in ten thousand was still infective. This same 100 µl sample (undiluted) would yield only ten plaques by plaque assay. However there would still be $10^5$ adsorbable units in the sample. If the number of receptors on the tissue culture cells were limited (eg. $10^4$), then there would be competition for the

available cell receptor sites. This would lead to the exclusion of some infectious particles, thereby causing a reduction in the number of plaques. In the above example, because of the high ratio of inactivated to infectious virions (10,000:1), it would be unlikely that any plaques would appear on a cell monolayer. Since the adsorption process is dependent on haemagglutinin and not on infectivity, there is not way of physically separating infectious and inactivated virions. The above hypothesis is, however, dependent on there being a limited number of receptor sites on a given cell monolayer.

The prozone effect might also be explained by the production of interferon by tissue culture cells, in response to the high concentration of virus in the inoculum. Further work, however, is required to elucidate the mechanisms involved in this phenomenon. The prozone phenomenon does, however, point out the necessity of performing an adequate number of dilutions in the plaque assay, especially when no plaque forming units are observed at stock concentration. If this is not done, a gross underestimation of sample infectivity could occur.

The infectivity of Influenza A/PR8 may be overestimated as well. This is due to the nature of the genetic material of Influenza A/PR8. This virus has been shown to have a single stranded RNA genome which is present in the virion as eight segments (Pons, 1975). It is possible that if one virion were inactivated on one segment and another virion inactivated on a different segment, multiple infection of a cell by these two virions would lead to an infection and therefore produce a plaque. However, the chances of this happening would seem to be very small and the incidence difficult to calculate.

## CONCLUSIONS

The trials of Influenza A/PR8 inactivation, described in this report,

support the current literature by showing that a significant drop in infectivity of psoralen and LWUV treated virus occurs without loss of antigenicity. This is due to the interaction of AMT and long wave ultraviolet light, since neither was able to significantly affect infectivity alone. The use of this method for vaccine production of Influenza A/PR8 shows promise. However, the possible side effects of psoralen and its derivatives (such as gene aberrations, carcinogenicity and toxicity) need to be studied (Wasserman, 1978).

It is now possible to move on to the next stage of this project, that is, monitoring of the immune response in mice following immunization with psoralen inactivated and live attenuated influenza vaccines.

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## APPENDIX

### Plaque Assay Overlay Medium

| | | |
|---|---|---|
| 1) Liquid phase: | 20.0 ml | 10X Auto-Pow minimum essential medium Eagle (modified) (MEM) without glutamine, without sodium bicarbonate (Flow Laboratories, Rockville, Maryland) |
| | 34.0 ml | supplement without sodium bicarbonate (see below) |
| | 1.2 ml | 0.1% trypsin TPCK (Worthington Biochemical Corporation, Freehold, New Jersey) in triple distilled water |
| | 38.8 ml | sterile triple distilled water |
| | 6.0 ml | 7.5% $NaHCO_3$ (Fisher Scientific Company, Fairlawn, New Jersey) |
| 2) Agar phase: | 1.6 gm | ionagar no. 2 (Oxoid, Med-Ox Chemicals, Ottawa, Ontario) |
| | 100 ml | triple distilled water |

Liquid and agar phases are mixed in equal volumes and held at 42°C prior to overlaying the plates.

Supplement without sodium bicarbonate:

| | |
|---|---|
| 20.0 ml | penicillin/streptomycin solution (penicillin-G 100 I.U./ml; streptomycin sulphate 200 µg/ml) |
| 20.0 ml | 200 mM L-glutamine (Flow Laboratories, Rockville, Maryland) |
| 60.0 ml | 100X MEM Vitamin solution (Gibco Laboratories, Grand Island, New York) |
| 240.0 ml | triple distilled water |

## Maintenance of Cell Cultures

MEM for African Green monkey kidney (Vero) cells:

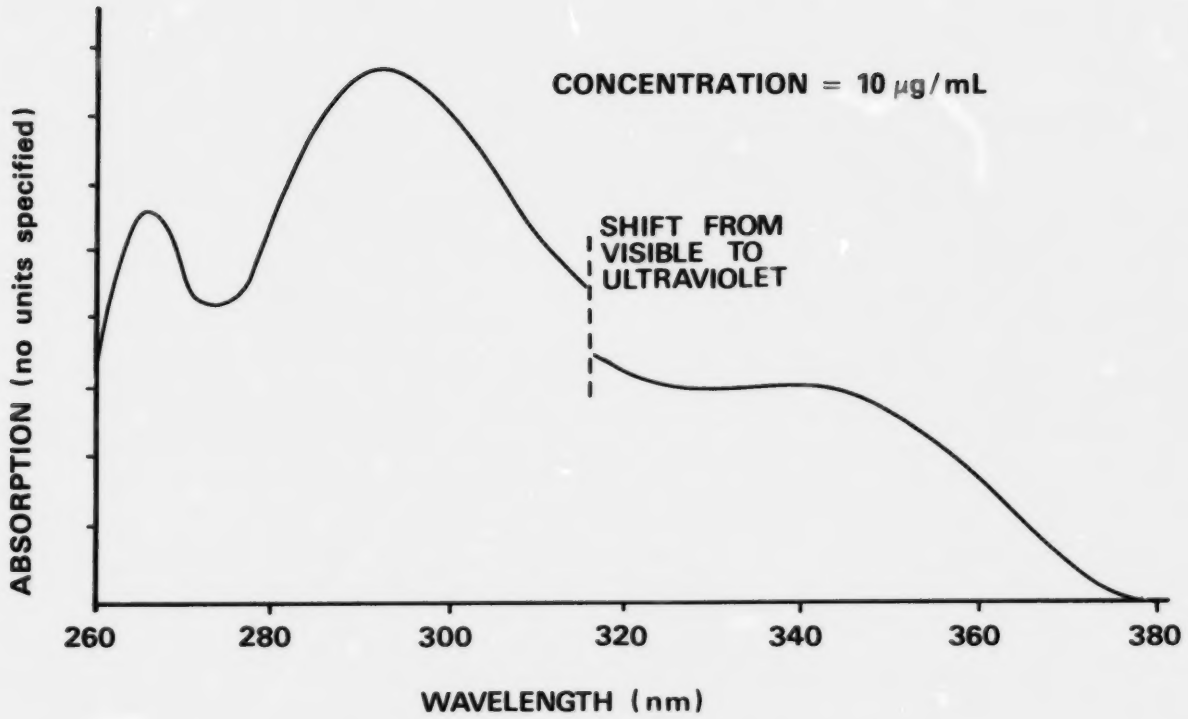
| | |
|---|---|
| 500.0 ml | 1X MEM without glutamine, without sodium bicarbonate (Cat. No. 11-100-22, Flow Laboratories, Rockville, Maryland) |
| 5.0 ml | penicillin/streptomycin solution |
| 5.0 mM | L-glutamine (Flow Laboratories, Rockville, Maryland) |
| 15.0 ml | bobby calf (newborn calf) serum (Gibco Laboratories, Grand Island, New York) |
| | 7.5% sodium bicarbonate, sufficient to turn the phenol red indicator orange-red |

Vero cell cultures are grown in 150 ml Corning cell culture flasks (Corning Glass Works, Corning, New York) with 50 ml of medium (see above) per flask. When monolayers become confluent, cells are trypsinized with 2X trypsin (Gibco Laboratories, Grand Island, New York) in PBS. This involves discarding the old medium, washing the cell sheets with approximately 25 ml of PBS and then incubating the cells with 2.0 ml of 2X trypsin at 37°C for approximately ten minutes, or, until the cells are removed. The cells are pipetted up and down in a small volume of maintenance medium and then, divided between two or three 150 ml cell culture flasks, or, ten plaque assay plates. Sufficient medium must be added to these cells such that each flask contains 50 mls MEM and each plaque assay plate contains 5.0 ml. For standardization all cells to be used for plaque assay plate production are pooled and dipensed (final cell concentration 1.0 - 2.0 x $10^6$/ml).

## Major Outbreaks of Influenza A in the Twentieth Century

| | |
|---|---|
| 1918 to 1919 | Strain related to swine influenza. Causes a particularly strong infection in young people. Most severe outbreak in recorded history. Mortality estimated at 20 to 50 million. |

| | |
|---|---|
| 1933 | Strain A/NWS/33 (HON1). First influenza isolation. |
| 1947 | Strain A/FM/1/47 (H1N1). |
| 1957 | Strain A/Singapore/1/57 (H2N2). Most severe outbreak with respect to morbidity. Low mortality. |
| 1968 | Strain A/Hong Kong/1/68 (N3N2). |




ABSORPTION SPECTRUM OF AMT (10 μg/mL) DISSOLVED IN DMSO

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4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

5 AUTHOR(S) (Last name, first name, middle initial)

S.H. Toenjes, R.E. Fulton, L.M. Mofford, J. Fildes and V.L. DiNinno

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13. ABSTRACT

A procedure which utilizes the property of 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) to bind to single stranded RNA in the presence of long wave ultraviolet light (LWUV) has been developed for the inactivation of influenza A/PR8. At a concentration of AMT of 10 µg/ml, inactivation was complete after an exposure time to LWUV of three min. Virus infectivity was not significantly affected by treatment with either AMT or LWUV alone. The ability of influenza A/PR8 to haemagglutinate rooster erythrocytes was unaffected by AMT-LWUV treatment. This procedure provides an alternative to chemical inactivation procedures which can alter the antigenicity of virus preparations and thereby compromise their value as protective inmunogens.

**KEY WORDS**

Psoralen

Influenza A

Inactivation

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